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Original Article

P2X2 Receptor Terminal Field Demarcates a "Transition Zone" for Gustatory and Mechanosensory Processing in the Mouse Nucleus Tractus Solitarius

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Abstract

Peripheral gustatory neurons express P2X2 purinergic receptors and terminate in the rostral portion of the nucleus tractus solitarius (rNTS), but a relationship between the P2X2 terminal field and taste evoked activity has not been established. Additionally, a portion of somatosensory neurons from the trigeminal nerve, which are devoid of P2X2 expression, also terminate in the lateral rNTS. We hypothesized that P2X2 receptor expression on afferent nerve endings could be used as an anatomical tool for segregating gustatory from mechanosensory responsive regions in the mouse rNTS. C57BL/6 mice were used to record extracellular activity from neurons within the rNTS and the laterally adjacent reticular formation and trigeminal nucleus. Histological reconstruction of electrolytic lesions indicated that gustatory activity coincided with electrode tracks that traversed through P2X2 terminal fields. Gustatory recordings made more rostral in the rNTS had receptive fields located in the anterior oral cavity (AO), whereas gustatory recordings made more caudal in the rNTS had receptive fields located in the posterior oral cavity (PO). Mechanosensory neurons with AO receptive fields were recorded near the lateral border of the P2X2 terminal field and became numerous on electrode tracks made lateral to the P2X2 terminal field. In contrast, mechanosensory responses with PO receptive fields were recorded within the P2X2 terminal field along with gustatory activity and transitioned to mechanosensory only outside the P2X2 terminal field. Collectively, our results indicate that the lateral border of the P2X2 terminal field, demarcates a faithful "transition zone," where AO responses transition from gustatory to mechanosensory.

Key words: gustatory, neurophysiology, NTS, P2X2, taste

Introduction

P2X2 and P2X3 are ionotropic purinergic receptor subunits co-expressed on peripheral gustatory afferents that form multimeric ionotropic receptors comprising a cation channel (Bo et al. 1999; Finger et al. 2005). ATP is released from taste buds cells during gustatory stimulation where it serves as a critical neurotransmitter for transmission of chemosensory signals to gustatory nerve fibers via interaction with these receptors (Finger et al. 2005; Huang et al. 2007; Vandenbeuch et al. 2015). This contention is supported by electrophysiological studies in mice, which demonstrate that double knockout of both the P2X2 and P2X3 subunits results in a complete loss of taste responses in the chorda tympani and glossopharyngeal

nerves, although knockout of either the P2X2 or P2X3 subunit alone just partially disrupts taste-mediated behaviors (Finger et al. 2005). Thus, P2X2/P2X3 expressing afferents play a pivotal role in transmitting gustatory sensations from taste buds to the central nervous system through the chorda tympani and glossopharyngeal nerves. Interestingly, these receptors are also expressed on the central endings of these nerves at their termination sites within the rostral nucleus tractus solitarius (NTS) (Bartel 2012; Ganchrow et al. 2014), suggesting there should be a relationship between the location of the P2X terminal field and taste responsivity in the nucleus. However, such a relationship has not been established.

Gustatory afferents arising from facial (VII) and glossopharyngeal (IX) nerves synapse onto somata and dendrites (Whitehead 1986; May et al. 2007) in the rostral portion of the NTS (rNTS), whereas the caudal portion of the NTS (cNTS) receives afferents from the vagus (X) nerve (Contreras et al. 1982; Hamilton and Norgren 1984). There is a rough, overlapping topography to the oral and laryngeal gustatory representation with gustatory afferents innervating taste buds in the anterior oral cavity (AO; nasoincisor duct and anterior tongue) located more rostrally in the rNTS, and those innervating taste buds in the posterior oral cavity (PO; foliate, circumvallate, and soft palate) pharynx and epiglottis located more caudally in the rNTS (Contreras et al. 1982; Hamilton and Norgren 1984; Travers and Norgren 1995; May and Hill 2006; Corson et al. 2012). Importantly, in addition to connecting to the more lateral trigeminal sensory complex, primary afferent somatosensory fibers also synapse in the rNTS, including several oral branches of the trigeminal nerve (V) (Contreras et al. 1982; Hamilton and Norgren 1984; Takemura et al. 1987, 1991) and somatosensory afferents traveling in the glossopharyngeal nerve (Frank 1991; Lasiter 1992).

P2X2 and P2X3 appear to be specifically expressed in intragemmal gustatory afferents (Bo et al. 1999; Finger et al. 2005), as lingual trigeminal nerve fibers are devoid of P2X2/3 immunoreactivity. Moreover, oral tactile and thermal responses in the glossopharyngeal are not impacted in P2X2/P2X3 double knockout mice (Finger et al., 2005). Thus, we hypothesized that immunohistochemical labeling of P2X-expressing fibers could serve as tool for identifying gustatory and mechanosensory responsive regions in the NTS. To address this question, we utilized neurophysiological mapping techniques in conjunction with immunohistochemistry to determine the relationship between the location and modality of the oral sensory response and the P2X2-terminal field in rNTS. Here, we show that the lateral border of the P2X2-terminal field, particularly in the rostral third of the mouse rNTS, demarcates a faithful "transition zone" where orosensory responses transition from gustatory to mechanosensory.

Materials and methods

Surgical preparation

All procedures were approved by the IACUC at Ohio State University. Data from these experiments are from 14 C57BL/6 mice (21–43 g). Mice were initially anesthetized with isoflurane and given a single intraperitoneal injection of urethane (1.0 mg/kg). Supplemental intraperitoneal injections of sodium pentobarbital (Nembutal, 10–25 mg/kg) were used to maintain a surgical level of anesthesia throughout the experiment. Mice were placed in a supine position and the hypoglossal nerves were cut bilaterally to eliminate tongue movements during taste application and/or mechanical stimulation of the oral cavity. Mice were tracheostomized with PE-50 or PE-60 tubing to prevent taste solutions from entering the airway. Sutures were placed bilaterally in the upper and lower lips and around the lower incisors to facilitate opening of the oral cavity for ease of taste delivery and for determining the location of the gustatory or mechanosensory receptive field.

Mice were secured in a nontraumatic head holder with blunt ear bars (45° taper). With the aid of a dissecting microscope, a thin suture was placed rostral to the foliate papillae to facilitate taste delivery to these taste buds, which are buried in trenches, as described previously in rats (Halsell et al. 1993; Travers and Norgren 1995; Geran and Travers 2006). A midline incision on the skin overlying the cranium was made and connective tissue was removed on the surface of the skull. Bone was gradually planed away with a dental burr caudal to lambda to allow access to the cerebellar surface.

Neurophysiology and orosensory stimuli

Glass-insulated tungsten electrodes (Z = 450 KΩ—1.3 MΩ) were used to record single-or multi-unit activity and were lowered into the cerebellum at a 0° or 10° pointed caudally with stereotaxic coordinates between 5.2—6.2 mm caudal and 0.6—1.8 mm lateral to Bregma. A reference ground was attached to either a skull screw, cortical electrode, or stereotaxic apparatus, depending on which configuration resulted in the least electrically "noisy" waveforms. Waveforms were amplified 10 000×, fed through an audiomonitor, viewed on an oscilloscope and computer monitor, and recorded to a hard drive online via Spike 2 software and hardware (Cambridge Electronic Design, UK). The electrode was advanced ventrally though the cerebellum to the brainstem with a hydraulic micromanipulator (David Kopf Instruments, Model# 640).

After monitoring the electrophysiological transition out of the brainstem vestibular nuclei (generally around 3800–4300 µm from cerebellar surface), we tested for orosensory responses. This transition is marked by decrease in spike amplitude and spike synchrony (which can be observed visually via oscilloscope and Spike2 software and audibly through our audio monitor), as the neurons in the NTS are much smaller than those in the vestibular nucleus. Mechanosensory receptive fields were located and activated by applying gentle pressure to the tongue, hard palate, soft palate, buccal wall, lower lip and teeth with blunt-glass probe or fine brush. Gustatory activity was evoked by applying a taste mixture (0.3 M sucrose, 0.1–0.3 M NaCl, 0.01–0.03 M citric acid, and 10 µM cycloheximide) and/or single components of the mixture, into the oral cavity with a 1 mL syringe or with a custom fluid-delivery system at a flow rate of 225 µL/s. Gustatory-receptive fields were determined by using a minute amount of the taste mixture or single taste stimulus on a fine brush. All taste compounds were reagent grade and purchased from Sigma or Fisher. The oral cavity was flushed with either artificial saliva (Breza et al. 2010) or distilled water before and after taste delivery to establish pre- and post-stimulation baseline activity and to determine whether neural responses were bona fide taste signals (tonic chemosensory activity) or due to tactile or thermal stimuli. Electrode tracks and physiological responses were monitored online with an oscilloscope and Spike2 hardware and software. Physiological responses to tactile and gustatory stimuli were recorded in a laboratory notebook and in some cases waveform data were saved and further reviewed post hoc.

After characterizing the multiunit or single unit activity at one or more NST sites along a track, the electrode was advanced ventrally; typically ~200–300 µm below the ventral border of taste activity, where only "jaw-stretch responses" (i.e., responses evoked by depressing the mandible) were present, suggesting that the electrode was in the reticular formation. Electrolytic lesions were made by passing negative current (3–8 µA for 3–5 s). In 8 cases, a second lesion was made in the vestibular nucleus to triangulate the lesions

with the recording sites. This second lesion in the vestibular nucleus was generally made on the electrode track where taste was first located. In a few cases, we made the lesion in the rNTS (n = 3) or in the trigeminal nucleus (n = 1) at the recording site. The electrode was then retracted out of the brain and positioned medially or laterally in 200–400 µm increments and the mapping procedure was repeated.

Tissue processing and immunohistochemistry

At the end of the experiment, mice were perfused intracardially with room temperature phosphate buffered saline (PBS, pH 7.4), followed by ice cold PLP, which consisted of 0.1 M phosphate buffer (PB), 4% paraformaldehyde, 1.4% l-lysine acetate and 0.2% sodium metaperiodate (McLean and Nakane 1974). Brains were extracted from the skull and a tungsten pin inserted through the left side of the brainstem to differentiate the right from left sides. Brains were then placed in a 20% sucrose solution made from the same PLP solution used during the perfusion, and post-fixed overnight. The brain was blocked the next day at a 0° or 10° angle, depending on which angle was used to advance the electrode through the tissue, and sectioned on a freezing microtome at 40 µM in a coronal plane. In cases where tissue sections were not immediately processed for immunohistochemistry, they were stored at –20 °C in cryoprotectant (Watson et al. 1986).

The entire medulla was sectioned starting from the most rostral portion of the decussation of the pyramids to the genu of the facial nerve. Tissue sections were thoroughly rinsed in PBS, before and after treatment of 1% sodium borohydride (20 min) and 0.5% H_2O_2 (peroxidase quench). Tissue sections were treated for 60 min with 0.3% Triton, 1%BSA, and 5% Donkey Serum prior to incubation with a rabbit primary polyclonal antibody (Alamone Labs, Catalog# APR-003, Lot# AN-05) generated against the intracellular C-terminus of the P2X2 receptor (1:10 000×) for 48–72 h at 4 °C. Tissue sections were then thoroughly rinsed in PBS and incubated in biotinylated rabbit secondary antibody (1:500×; Jackson ImmunoResearch Laboratories, Inc.) for 1.5 h at room temperature and then in an avidin-biotin mixture (Elite Kit, Vector) diluted in 0.1 M PB and 1% BSA. The final chromagen reaction began with incubation in 0.05% 3, 3′-diaminobenzidine-HCl (DAB) with 0.016% nickel ammonium sulfate, followed by the oxidation stage, initiated by adding H_2O_2 sufficient to achieve a concentration of 0.003%. This concentration of nickel labeled P2X2 afferents dark brown. In a few cases (N = 4), one series of tissue was double-labeled for NeuN, a neuronal marker, (mouse primary Millipore, Lot# LV1825845) using DAB without nickel to produce a light brown reaction product, and for P2X2 with 0.12% nickel ammonium sulfate to produce a black reaction product. For the remaining series, immunofluorescence was used to stain for P2X2 (rabbit secondary antibody; 1:500×, Alexa Fluor 488).

Data analysis and photomicroscopy

Photomicrographs (10×) of tissue sections were captured under darkfield, which allowed us to view the borders of the poorly myelinated rNTS and facilitated locating the center of electrolytic lesions. In most cases, the P2X2 labeling was robust enough to view under darkfield, but brightfield photomicrographs (10×) of the same sections were captured to optimally view the location of the electrolytic lesion in relationship to the P2X2 terminal field. Electrolytic lesions were plotted on 3 representative sections of the rNTS, spanning 3 different levels along the rostro-caudal axis, similar to our earlier representations of the rat rNTS (Travers 2002). The sections are referred to as r1–r3. In the present manuscript, r1 is the caudal limit of the rNTS, and r2 and r3 are more rostral locations separated by approximately equal intervals. The predominant afferent terminations in r3 are from gustatory fibers in the VIIth nerve, which innervates taste buds on the anterior tongue (fungiform), nasoincisor duct, and soft palate. In contrast, r1 and r2 (in the rat) receive gustatory and mechanosensory information mainly from the IX nerve, which innervates taste buds in the circumvallate and foliate papillae (Lundy and Norgren 2015).

Results

Shown in Figure 1 is a horizontal section of the mouse brainstem, immunohistochemically processed for the P2X2 receptor. The section from this animal was used solely for anatomical purposes—no electrodes were advanced through the

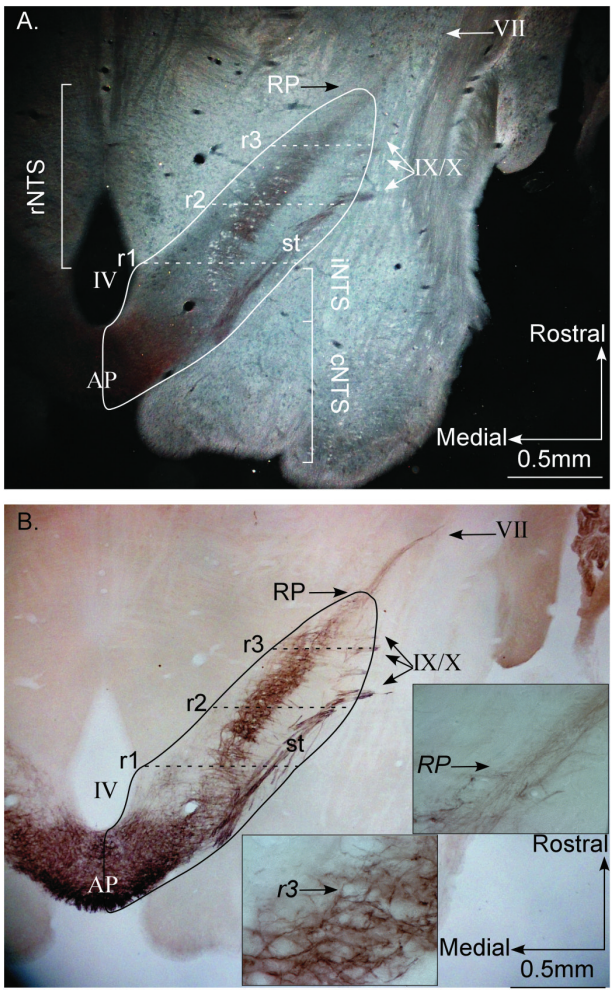

Figure 1. The P2X2 receptor is highly expressed on afferent-nerve fibers throughout the rostral to caudal extent of the mouse NTS and area postrema. Photomicrographs (**A**: darkfield and **B**: brightfield) of the mouse brainstem, cut in a horizontal plane (12° angle relative to the cutting plane, rostral tilted up) and immunohistochemically labeled for the P2X2 receptor. P2X2 positive fibers are labeled brown (DAB). The NTS borders are outlined in white (A) and black (B). The rNTS extends from the rostral pole (RP) of the nucleus to the point at which the NTS first separates from the IVth ventricle (labeled r1 IV). Three representative levels equally spaced throughout the rNTS are indicated by dotted lines (r1–r3). Level r3 receives gustatory input primarily from the facial (VII) nerve; r2 and r3 mainly from the glossopharyngeal (IX) nerve. The intermediate NTS (iNTS) extends caudally to the rostral pole of the area postrema (AP), and the caudal NTS (cNST) includes the remainder of the NTS that is caudal to iNTS. The insets in B show the rostral pole and r3 regions at a higher magnification; inset width = 230 µm.

brain. Photomicrographs of the same section were taken under darkfield (A) to highlight the borders of the NTS and brightfield (B) to show the P2X2 fibers and to indicate locations of the representative sections used to plot the recording sites in Figure 2. P2X2 fiber staining was abundant in the area postrema (AP) and throughout the caudal and rostral extent of the NTS, both in the solitary tract (ST) and in the nucleus itself, where the fibers become finer and more varicose, most likely indicative of primary afferent terminations. Outside the borders of the nucleus, the VIIth, IXth, and Xth cranial nerves enter the brainstem from the lateral aspect and travel as distinct bundles toward the NST. All recordings were made in the rNTS.

Figure 2 shows photomicrographs of coronal sections from the mouse medulla immunohistochemically stained for the P2X2 receptor. These 3 P2X2 labeled sections defined as r1, r2, and r3 served as the representative sections for plotting all the recording tracks in the current study. Sections were taken from a single case (#12) used for recording, and the electrolytic lesions made in this animal are apparent in Figure 2, section r2. Each dotted line corresponds to a track and the type of symbol indicates the response modality encountered

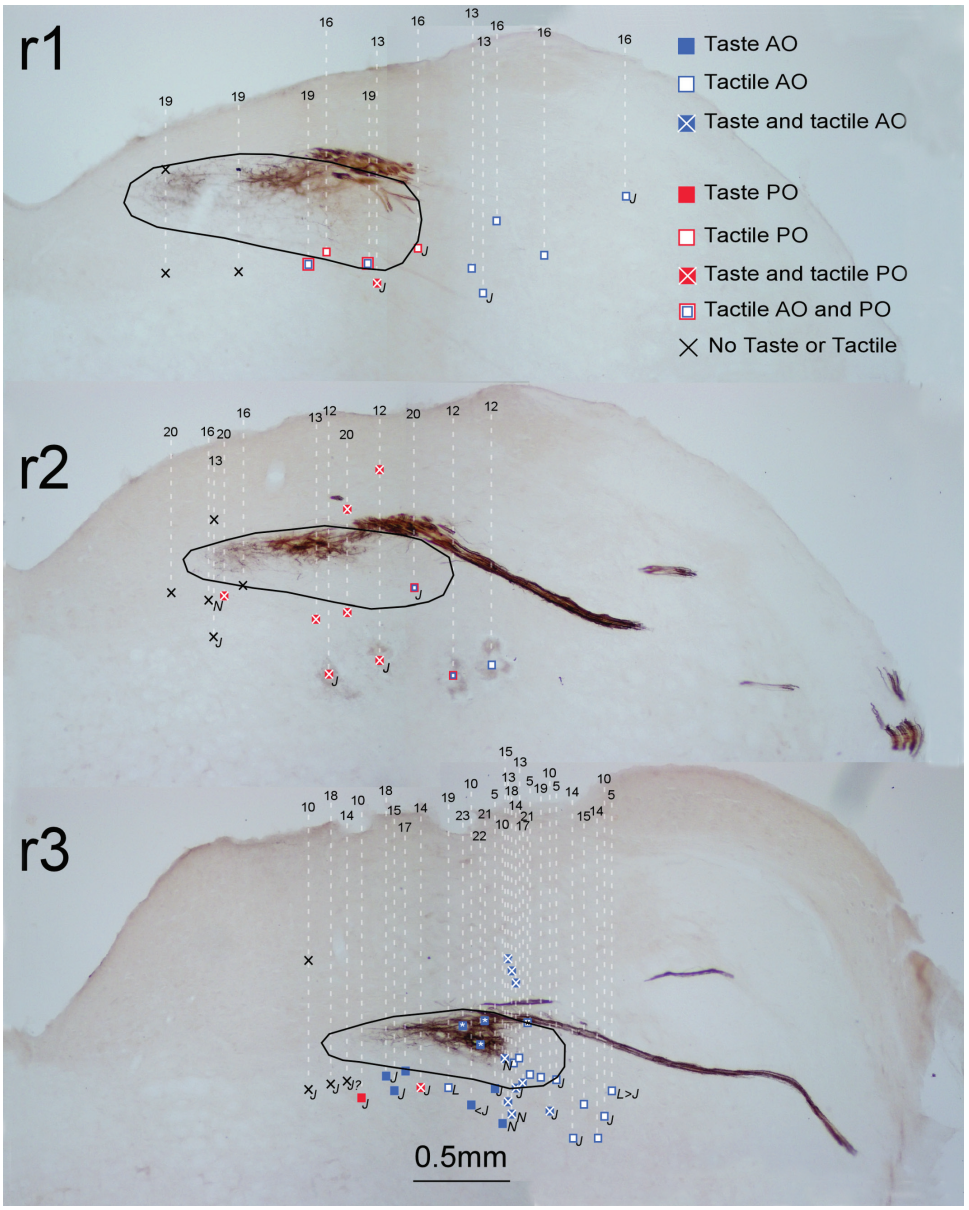

Figure 2. Response modality and receptive field vary systematically with P2X2 terminal field and rNTS location. Photomicrographs of P2X2 stained sections (coronal plane) from the 3 levels of the mouse rNTS (r1–r3) indicated in Figure 1 summarize all recording tracks from each subject in this study. Borders were outlined by referring to both the darkfield and brightfield images. The trajectory of each recording track (white dotted lines) is summarized. The numbers at the top of the tracks indicate the mouse numbers. Symbols indicate the modality of the physiological response recorded in the NTS or at a similar depth lateral to the nucleus. The locations of the symbols indicate the locations of the lesions which were typically made ventral to the recording site in the rNTS. However, in a few cases, lesions were made both dorsal and ventral to the recording site or at the recording site (symbols with "*"). The letters adjacent to the symbols indicate the responses recorded at the ventral lesion sites. These photomicrographs were taken from case #12 and electrolytic lesions can be seen on r2. AO, anterior oral cavity; PO, posterior oral cavity; N, no response; J, response to depressing the mandible [jaw]; L, response to tapping the lower incisor; <, the noted response was small; ?, the noted response was questionable; blank, no information.

in the NTS. The locations of the symbols, however, indicate locations of the lesions, which were usually made ventral to the nucleus and the responses that they denote. Letters adjacent to the symbols refer to responses recorded at the lesion site itself. Most of the lesions were made in the ventral subnucleus or reticular formation and these sites were often responsive to depressing the mandible. It is notable that, similar to the limited extent of the P2X2 staining apparent in the dorsoventral axis, gustatory responses extended just a short distance in the dorsoventral axis along a given track. Indeed, for those tracks where sufficient information was available (n = 10), on average, gustatory responses could be recorded for just 191 ± 33 µm.

Gustatory responses at r3 (Figure 2) were confined to the P2X2 terminal field, and were almost exclusively (one exception) from the AO (fungiform papillae and nasoincisor ducts). Physiological responses near the lateral border of the P2X2 terminal field marked a "transition zone" where multiunit responses arose from both taste and mechanosensory stimulation but were exclusively from the AO. Lateral to this "transition zone," responses were purely mechanosensory and were exclusively from the AO, including the anterior tongue, hard palate, and buccal wall. Responses on one track, made lateral to the P2X2 terminal field, was a mixture of gustatory and mechanosensory responses to stimulating the AO (specifically the anterior tongue and hard palate), but this gustatory response was weak and possibly recorded from the solitary tract.

At r2 (Figure 2), most responses aligned with the P2X2 terminal field had gustatory inputs but they were invariably accompanied by equally or more robust mechanosensory responses from the PO. Most of these responses arose from the foliate or circumvallate papillae or from the soft palate, but occasionally from posterior fungiform papillae. One gustatory-responsive site was encountered just outside the lateral border of the P2X2 terminal field, but the characteristics of this response (mechanosensory and gustatory) did not differ from those made within the terminal field. Other orosensory responses recorded outside the P2X2 terminal field were purely mechanosensory and had AO, AO and PO, or PO inputs. Moreover, mechanosensory posterior tongue responses encountered lateral to the P2X2 terminal field did not arise specifically from the foliate or circumvallate papillae but often responded to other regions of the posterior tongue.

At level r1 (Figure 2), the correspondence between taste responses and the P2X2 terminal field was less obvious, but fewer tracks were made through this level and none were made through the region of the strongest P2X2 terminal field staining. Nevertheless, the 2 most medial tracks passed through a region of fine P2X2 varicosities and no orosensory responses were encountered. At the lateral edge of the P2X2 terminal field, and more laterally where P2X2 staining was associated with the solitary tract, the majority of the responses were tactile and arose from the PO or both the AO and PO.

Figure 3 shows an example illustrating the general pattern of the gustatory to tactile transition in a case (#21) where lesions were made at (not ventral to) single-unit recording sites. One lesion was made within the P2X2 terminal field and another just lateral to it, as apparent in the darkfield photomicrograph with superimposed P2X2 immunofluorescence (Figure 3A). The site within the P2X2 field responded to gustatory stimulation and had a receptive field confined to the anterior tongue (Figure 3B) whereas the more lateral site responded to mechanical stimulation of the buccal wall (Figure 3C). An interesting instance of the lateral to medial transition from mechanosensory to gustatory responses is depicted in Figure 4. On the lateral track, which was well outside the P2X2 field in the spinal trigeminal nucleus, 2 mechanosensory neurons were recorded at a single location, one responsive to stimulation of the anterior buccal wall and the other to the anterior tip of the tongue. On the more medial track, which was on the lateral fringe of the P2X2 terminal field, a single neuron was encountered that responded with robust tonic activity to the taste mixture and to mechanical stimulation of the anterior tip of the tongue. Gustatory activity was more robust than mechanosensory activity for this neuron, but there were a few smaller neurons (see raw trace) that responded only to mechanosensory stimulation. Figure 5 illustrates a final example of the coincidence of gustatory responsiveness and P2X2 labeling from a case (#22) double-stained with NeuN and P2X2 (Figure 5A) where a lesion was made at a multiunit site responsive to taste stimulation of the anterior tongue (Figure 5B). The denser cell body packing characteristic of the rostral central subdivision is apparent at the edges of the P2X2 label and contrasts with the sparser packing in the medial, rostral lateral, and ventral subdivisions.

Discussion

The purpose of this investigation was to determine whether the P2X2 terminal field could be used as an anatomical guide to demarcate an orosensory-transition zone in the rNTS, where orosensory responses change from gustatory to mechanosensory in a medial to lateral direction, respectively. To address this issue, we recorded neurophysiological responses in the mouse medulla within and near the rNTS, employed electrolytic lesions, and used immunohistochemical methods to identify P2X2 expressing nerve fibers. As shown in the summary figure (Figure 2), the majority of lesions were made below the rNTS. We chose to make most lesions outside the nucleus, since electrolytic lesions disrupt immunohistochemical staining and because we wished to garner multiple data points from individual mice without compromising circuitry within the nucleus. Because orosensory responses were first encountered just ventral to the obvious neurophysiological transition from the larger to the smaller amplitude neural activity characterizing the transition from the vestibular to the solitary nucleus, and because taste responses extended a maximum of just a few hundred microns, it seems likely that responses aligned with the P2X2 staining were mainly recorded within, not ventral to the P2X2-positive region. This contention is supported by the cases in which lesions were made at the recording sites (Figures 2, 3, and 5) and by those instances where lesions above and below the recording site optimized accurate estimation of its location (Figure 4).

Structure and functional relationship of P2X2 terminal fields in the rNTS

The novel finding in the present investigation is that the lateral edge of the P2X2 terminal field demarcates a transition zone from gustatory to oral mechanosensory responses. This transition zone was clearest in the region most thoroughly explored, the rostral rNTS (r3) where AO responses predominate. At this level, gustatory responses, with minimal or no accompanying tactile responses, were recorded throughout the P2X2 terminal field, whereas mechanosensory responses originated near the lateral border of the P2X2 zone and continued laterally outside the nucleus. These neurophysiological results complement the elegant anatomical work of Bartel (2012) who demonstrated that immunohistochemistry for P2X2 (and P2X3) co-labeled chorda tympani afferents in this region of the rNTS. At the r3 lateral transition zone, AO gustatory responses were invariably accompanied by AO mechanosensory responses and, similar to the rat (Travers and Norgren 1995), these mechanosensory

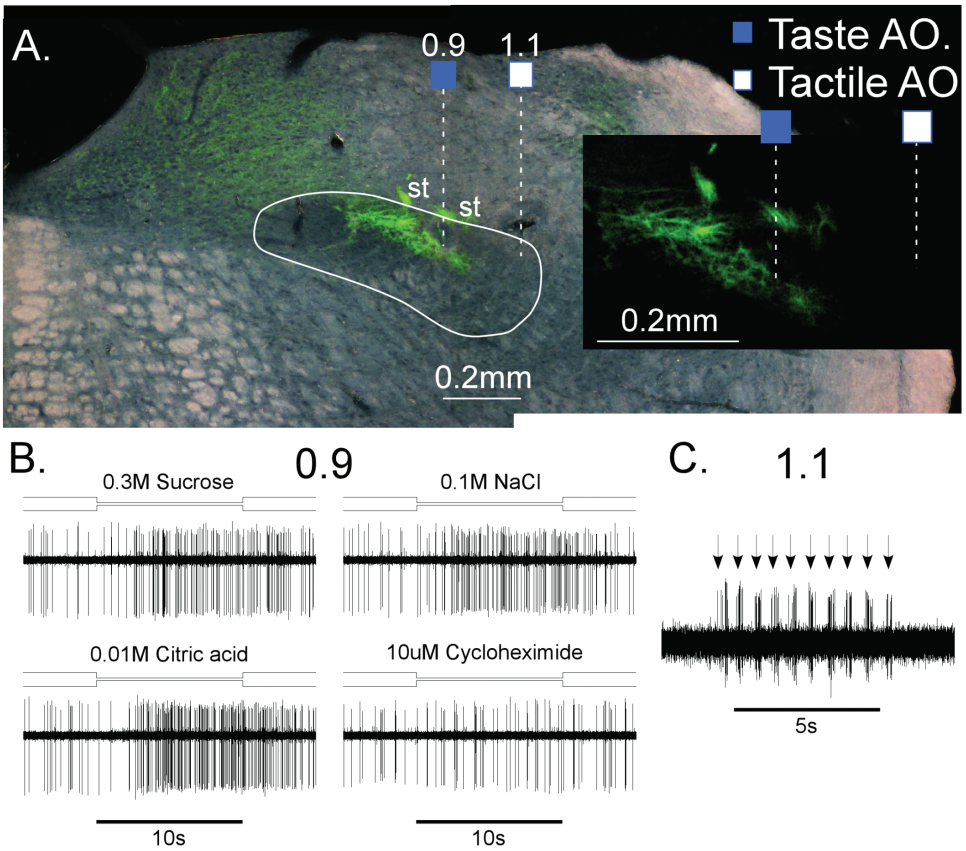

Figure 3. Example illustrating that the locations of rNTS taste and tactile responses vary systematically in the mediolateral axis and in relation to the P2X2 terminal field. (**A**) Darkfield photomicrograph of a coronal section from the rNTS (r3; case #21) with superimposed P2X2 immunofluorescence. The borders of the rNTS are outlined in white. The solitary tract (st) travels in and out of the plane of the coronal section, making it appear as 2 separate fiber bundles. The dotted lines indicate the electrode trajectory and end at the ventral limits of the lesions, apparent in the photomicrographs. Our prior experience suggests that recording sites are closest to this ventral limit. Symbols representing the types of physiological responses recorded at the lesion sites and coordinates relative to midline are presented at the top of the track. The P2X2 immunofluorescence is enlarged without darkfield superimposed (top right) to highlight the lesion locations relative to the P2X2 terminal field. A gustatory neuron, recorded within the P2X2 terminal field (**B**; 0.9 mm), responded broadly to stimuli representing the 4 basic taste qualities. Raw neurophysiological data are shown for each taste stimulus, which were presented for 10 s. Square waves at the top of each trace indicate when the fluid valves switched from rinse to taste stimulus. A mechanosensory neuron was recorded (**C**) outside of the P2X2 terminal field on a more lateral track. Arrows indicate the approximate time at which the glass probe made contact with the buccal wall. AO, anterior oral cavity.

responses were less vigorous than gustatory responses (Figure 4) with our chosen stimulus parameters, which were midrange for chemosensory stimuli (Frank 1973; Frank et al. 1983; Danilova and Hellekant 2003; Eylam et al. 2003; Eylam and Spector 2004; Dotson et al. 2005; Glendinning et al. 2005). We cautiously interpret these findings as evidence that our recording electrode was closer in proximity to gustatory neurons, but only a dose–response relationship of mechanical force and taste-stimulus concentration could provide complete picture of the chemosensory to mechanosensory continuum.

Even further laterally, orosensory responses were purely mechanosensory and arose from the AO. A similar pattern was observed at a mid-level of the rNTS (r2), where PO responses predominated. However, at the mid-level, gustatory responses were always intermingled with robust mechanosensory responses within the P2X2 field. In addition, although purely mechanosensory responses were found lateral to the mixed taste/mechanosensory responses, most of the lateral tracks were actually outside the rNTS and thus, the rNTS region lateral to the P2X2 afferents was not well-characterized. At the most caudal level of the rNTS (r1), only one gustatory-responsive track was encountered and thus no transition between modalities could be observed. However, fewer tracks were made through this rNTS region and only 4 appeared to pass through the P2X2 field. Although we attempted to map throughout the nucleus, the final sample was largest for the most rostral levels, in part reflecting the fact that responses were weaker and more difficult to characterize caudally. Thus, conclusions are premature for the more caudal levels. However, based on the critical function of the P2X2 receptor for taste but not tactile responses in both the chorda tympani and glossopharyngeal nerves (Finger et al. 2005), it would not be surprising if denser sampling at the more caudal levels revealed the same pattern.

Our physiological results throughout the rostral to caudal extent of the rNTS are in general agreement with mapping studies of the rat rNTS (Halsell et al. 1993; Travers and Norgren 1995). Travers and Norgren (1995) found that gustatory neurons in the rostral third of the rat rNTS, dubbed r3 in the current mouse investigation, were rostral and medial to mechanosensory responses. Moreover, rat rNTS gustatory neurons at a level consistent with the mouse r3, generally had AO receptive fields, but neurons with PO receptive fields were located more medial to AO responses and at the caudal end of this level. In the present investigation, we also recorded from a more medial gustatory neuron with a PO receptive field at r3, and subsequent unpublished observations in our lab suggest that this may be a general pattern.

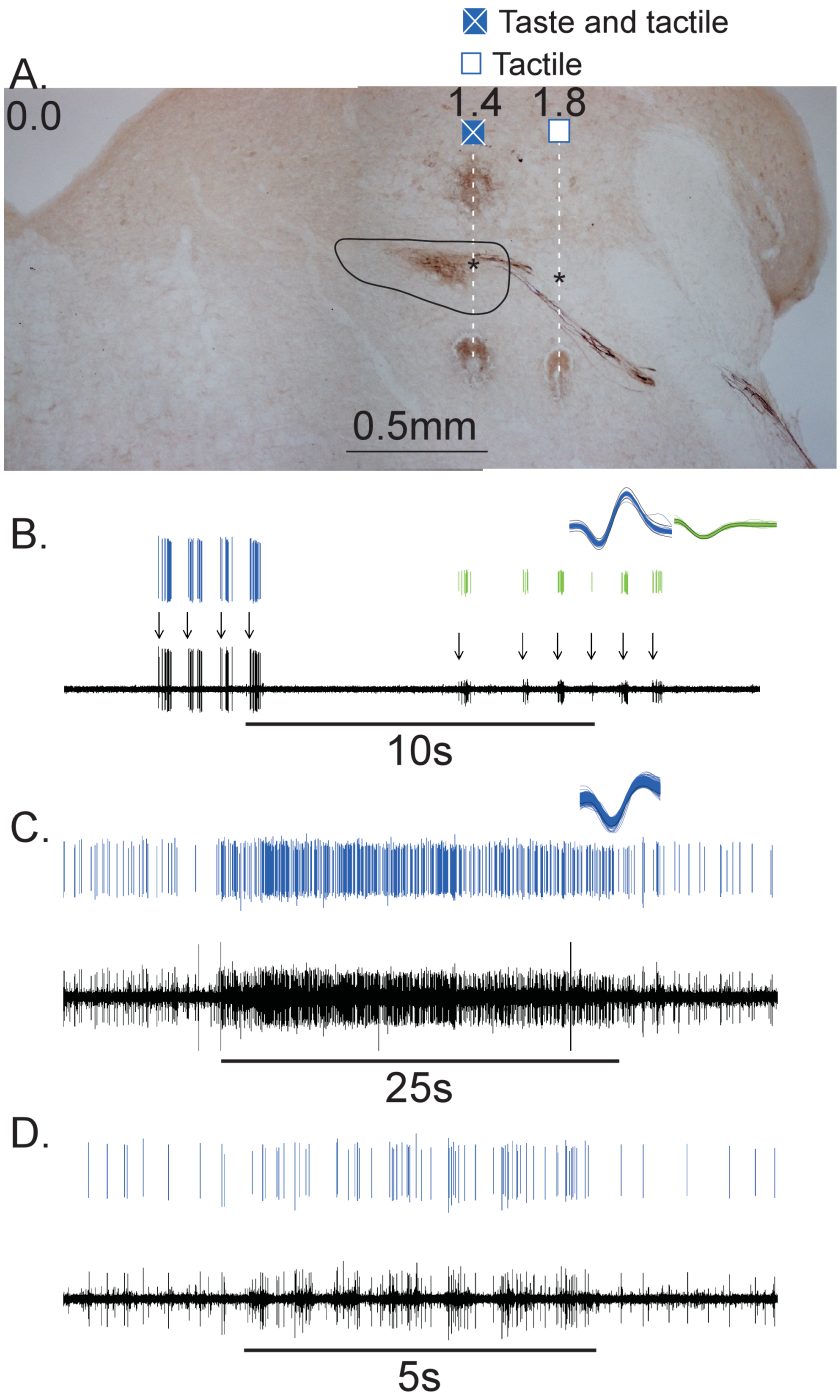

Figure 4. Example illustrating mixed taste/orotactile responses on the lateral border of the P2X2 terminal field in contrast to a more lateral site with purely tactile responses. Photomicrograph (**A**) of a coronal section from the rNTS (r3, case #15) immunohistochemically labeled for P2X2 where lesions on the medial track were made both above and below the medial recording site, allowing more accurate reconstruction of the depths of the recorded responses. The trajectories of the tracks are represented by the dotted line and estimated locations of the recording sites are indicated by "*." Symbols representing the sensory responses at the estimated recording sites and coordinates relative to midline are presented at the tops of the electrode tracks. AO mechanosensory responses from 2 neurons (see waveform templates in blue and green) were recorded lateral to the P2X2 terminal field (**B**; 1.8 mm relative to midline) at a single location; arrows indicate when the glass probe made contact with the AO receptive fields. The receptive field for the large neuron (blue template) was on the anterior buccal wall, whereas the receptive field for the small neuron (green template) was on the anterior tongue. AO responses at the transition zone were both gustatory (**C**) and mechanosensory (**D**), though gustatory responses were more robust. The taste stimulus (taste mixture) was applied to the mouth with a 1 mL syringe, and stayed on the receptor surface for ~20 s before rinsing with artificial saliva. Stimulus artifacts from manually applying the stimulus and rinse can be seen in the raw trace. A single neuron was isolated from the neural activity (see template in blue), which responded better to the taste mixture than to mechanical stimulation of the AO (D), but several smaller neurons (see raw trace) were mechanosensitive.

Gustatory responses recorded from r2 had PO receptive fields and were invariably accompanied by mechanosensory responses from the PO, consistent with the rat (Halsell et al. 1993). These results are also in agreement with neuroanatomical studies conducted in rodents, showing a rough rostral to caudal orotopy for terminations of afferents from the VIIth and IXth nerves (Contreras et al.

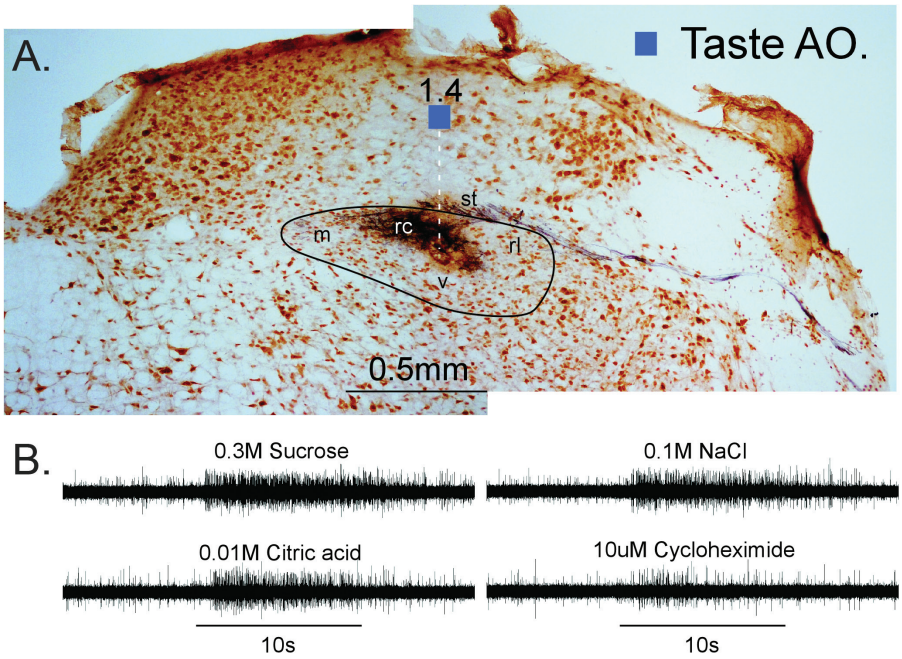

Figure 5. (**A**) Example illustrating the cytoarchitecture of the NTS from a case where gustatory responses were recorded within the ventral border of the P2X2 terminal field. Brightfield photomicrograph a coronal section from the rNTS (r3) (case#23) immunohistochemically labeled for NeuN and P2X2 receptors. Neurons labeled with NeuN are light brown, whereas P2X2 labeled fibers are black. A single electrode track (dotted line) ends at the ventral limit of the lesion, which was made within the P2X2 terminal field. The NeuN staining allowed identification of NTS subdivisions. The area occupied by the P2X2 fibers is the rostral central subdivision (rc) and the tight packing of small neurons characteristic of this region can be viewed at the medial and lateral edges of the P2X2 staining. Both the rostral lateral (rl) and ventral subdivisions (v) are less densely populated. The medial subdivision (m) is even more sparsely populated and contains very small cells (*note*: the large preganglionic parasympathetic neurons in this region are known to stain poorly for NeuN (Mullen et al. 1992). The coordinate relative to midline and the symbol representing the physiological response are presented at the top of the track. Raw multicellular activity, with receptivefields from the anterior oral cavity (AO), were recorded (**B**) in response to stimuli representing the 4 basic taste qualities. The line at the bottom of the traces indicates when the fluid valves switched from rinse to taste stimulus.

1982; Hamilton and Norgren 1984; May and Hill 2006; Wang and Bradley 2010; Corson et al. 2012). On the other hand, the anatomical studies appear to show more overlap and convergence between different cranial nerves than was apparent in this and other physiological studies. Indeed, anatomical tracing routinely shows that a small population of chorda tympani afferents innervates more caudal regions of the rNTS and some even traverse to the cNTS (May and Hill 2006; Corson et al. 2012; Ganchrow et al. 2014). However, we did not record from gustatory neurons with AO receptive fields outside r3 of the rNTS.

Although it has been our experience in both rat and mouse that gustatory responses, either from the AO or PO, are increasingly difficult to record at more caudal levels (Travers and Norgren 1995) we are not convinced that this indicates a lack of gustatory function. Indeed, glossopharyngeal projections to these regions of the rNTS are robust, and activation of rNTS neurons by bitter stimuli peaks in the caudal rNTS, when measured by Fos immunohistochemistry (Harrer and Travers 1996; King et al. 2000; Travers 2002; Travers and Travers 2007). Thus, the sparse taste responses in recording studies such as the present one may to some extent reflect the more challenging recording conditions in the posterior regions including the apparently smaller size of PO-responsive gustatory neurons (Halsell et al. 1993; Geran and Travers 2006) and the difficultly of optimally stimulating the taste receptors housed in the trenches of the foliate and circumvallate papillae (Herness 1988; Frank 1991). It is also possible that afferent inputs to these regions have a more subtle role that would only be apparent with more complex stimulating paradigms or in awake, behaving animals.

Similarly, it was notable that we recorded very few responses medially, especially at levels R2 and R3. This was despite the presence of (weak) P2X2 staining and is puzzling in light of the previously mentioned observations of robust bitter-evoked Fos in or near to this region. We did not explore this area thoroughly but only one of the 6 tracks that passed through P2X2 staining in the caudal, medial zone was responsive to orosensory stimulation. The PO taste and tactile responses encountered at this site were consistent with our previous work in rat (Travers and Norgren 1995; Geran and Travers 2006), but the more extensive rat studies yielded a greater number of sensory responses. Thus, the medial pole of the rNTS remains enigmatic although a role in autonomic control seems plausible, based on the presence of preganglionic parasympathetic neurons (Contreras et al. 1980) and strong projections from both the central nucleus of the amygdala (Halsell 1998), and caudal NTS (Breza et al. 2013).

P2X2 expression

The P2X2 antibody strongly labeled afferent nerve fibers throughout the rostral to caudal extent of the NTS and area postrema, as reported by other investigators (Bartel 2012; Ganchrow et al. 2014). It seems likely that this fiber staining mostly represents solitary tract terminations, as strongly suggested by robust staining in the solitary tract itself (Figures 1 and 2) and directly verified for the chorda tympani nerve by Bartel (2012). Previous studies specified that, in the rNTS, this fiber labeling was most prominent in the rostral central subdivision (Bartel 2012; Ganchrow et al. 2014). The location of the P2X2 staining that we observed is consistent with these observations,

as were the subset of cases double-stained with NeuN (Figure 5), that allowed a more direct evaluation of cytoarchitecture. Thus, the medial to lateral transition of taste to somatosensory responses most likely corresponds to the anatomical transition between the rostral central and rostral lateral subdivisions. This would be consistent with the reported dominant projections of the chorda tympani and lingual nerves to the rostral central and lateral subdivisions, respectively (Corson et al. 2012). Interestingly, although the chorda tympani nerve also terminates in the rostral lateral subdivision, not only is the projection weaker but the fibers are coarser than those terminating in the rostral central subdivision (Wang et al. 2012), similar to morphological differences initially noted for the medial versus lateral components of the glossopharyngeal projection in rat (Lasiter 1992) and cat (Claps et al. 1989), providing anatomical support for a functional difference between these subdivisions. Notably, the dominant fiber staining in rNTS observed in this and 2 previous studies using the same antibody (Bartel 2012; Ganchrow et al. 2014) contrasts with an earlier investigation using an antibody directed against the extracellular, rather than the intracellular domain of the receptor, where somal staining in the NTS was very prominent (Yao et al. 2001).

In that investigation, somal staining in the NTS was pronounced (Yao et al. 2001) whereas it was not notable using the current antibody. Thus, ionotropic purinergic receptors may be distributed both pre- and post-synaptically. In any case, the function of ionotropic purinergic receptors in the rNTS is unknown. In the cNTS, *in vitro* neurophysiological studies have demonstrated that P2X agonists are excitatory. In fact, in the medial and central regions of the caudal and intermediate NTS, P2X agonists increased the frequency of spontaneous glutamatergic post-synaptic currents (Jin et al. 2004) and the amplitude of excitatory post-synaptic currents evoked by solitary tract stimulation (Babic et al. 2015) suggesting a purinergic enhancement of primary afferent input. Thus, it will be interesting to ascertain whether a similar excitatory function holds for the rostral, gustatory region of the nucleus.

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References

Babic T, Ambler J, Browning KN, Travagli RA. 2015. Characterization of synapses in the rat subnucleus centralis of the nucleus tractus solitarius. *J Neurophysiol*. 113(2):466–474.

Bartel DL. 2012. Glial responses after chorda tympani nerve injury. *J Comp Neurol*. 520(12):2712–2729.

Bo X, Alavi A, Xiang Z, Oglesby I, Ford A, Burnstock G. 1999. Localization of ATP-gated P2X2 and P2X3 receptor immunoreactive nerves in rat taste buds. *Neuroreport*. 10(5):1107–1111.

Breza JM, Chen Z, Travers JB, Travers SP. 2013. Phenotypic characterization of a caudal to rostral intrasolitary pathway. *Chem Senses*. 38.

Breza JM, Nikonov AA, Contreras RJ. 2010. Response latency to lingual taste stimulation distinguishes neuron types within the geniculate ganglion. *J Neurophysiol*. 103(4):1771–1784.

Claps A, Torrealba F, Calderón F. 1989. Segregation of coarse and fine glossopharyngeal axons in the visceral nucleus of the tractus solitarius of the cat. *Brain Res*. 489(1):80–92.

Contreras RJ, Beckstead RM, Norgren R. 1982. The central projections of the trigeminal, facial, glossopharyngeal and vagus nerves: an autoradiographic study in the rat. *J Auton Nerv Syst*. 6(3):303–322.

Contreras RJ, Gomez MM, Norgren R. 1980. Central origins of cranial nerve parasympathetic neurons in the rat. *J Comp Neurol*. 190(2):373–394.

Corson J, Aldridge A, Wilmoth K, Erisir A. 2012. A survey of oral cavity afferents to the rat nucleus tractus solitarii. *J Comp Neurol*. 520(3):495–527.

Danilova V, Hellekant G. 2003. Comparison of the responses of the chorda tympani and glossopharyngeal nerves to taste stimuli in C57BL/6J mice. *BMC Neurosci*. 4:5.

Dotson CD, Roper SD, Spector AC. 2005. PLCbeta2-independent behavioral avoidance of prototypical bitter-tasting ligands. *Chem Senses*. 30(7):593–600.

Eylam S, Spector AC. 2004. Stimulus processing of glycine is dissociable from that of sucrose and glucose based on behaviorally measured taste signal detection in Sac 'taster' and 'non-taster' mice. *Chem Senses*. 29(7):639–649.

Eylam S, Tracy T, Garcea M, Spector AC. 2003. Amiloride is an ineffective conditioned stimulus in taste aversion learning in C57BL/6J and DBA/2J mice. *Chem Senses*. 28(8):681–689.

Finger TE, Danilova V, Barrows J, Bartel DL, Vigers AJ, Stone L, Hellekant G, Kinnamon SC. 2005. ATP signaling is crucial for communication from taste buds to gustatory nerves. *Science*. 310(5753):1495–1499.

Frank M. 1973. An analysis of hamster afferent taste nerve response functions. *J Gen Physiol*. 61(5):588–618.

Frank ME. 1991. Taste-responsive neurons of the glossopharyngeal nerve of the rat. *J Neurophysiol*. 65(6):1452–1463.

Frank ME, Contreras RJ, Hettinger TP. 1983. Nerve fibers sensitive to ionic taste stimuli in chorda tympani of the rat. *J Neurophysiol*. 50(4):941–960.

Ganchrow D, Ganchrow JR, Cicchini V, Bartel DL, Kaufman D, Girard D, Whitehead MC. 2014. Nucleus of the solitary tract in the C57BL/6J mouse: subnuclear parcellation, chorda tympani nerve projections, and brainstem connections. *J Comp Neurol*. 522(7):1565–1596.

Geran LC, Travers SP. 2006. Single neurons in the nucleus of the solitary tract respond selectively to bitter taste stimuli. *J Neurophysiol*. 96(5):2513–2527.

Glendinning JI, Bloom LD, Onishi M, Zheng KH, Damak S, Margolskee RF, Spector AC. 2005. Contribution of alpha-gustducin to taste-guided licking responses of mice. *Chem Senses*. 30(4):299–316.

Halsell CB. 1998. Differential distribution of amygdaloid input across rostral solitary nucleus subdivisions in rat. *Ann N Y Acad Sci*. 855:482–485.

Halsell CB, Travers JB, Travers SP. 1993. Gustatory and tactile stimulation of the posterior tongue activate overlapping but distinctive regions within the nucleus of the solitary tract. *Brain Res*. 632(1–2):161–173.

Hamilton RB, Norgren R. 1984. Central projections of gustatory nerves in the rat. *J Comp Neurol*. 222(4):560–577.

Harrer MI, Travers SP. 1996. Topographic organization of Fos-like immunoreactivity in the rostral nucleus of the solitary tract evoked by gustatory stimulation with sucrose and quinine. *Brain Res*. 711(1–2):125–137.

Herness S. 1988. Gustatory stimulating technique for glossopharyngeal neurophysiological recordings. *J Neurosci Methods*. 3:237–242.

Huang YJ, Maruyama Y, Dvoryanchikov G, Pereira E, Chaudhari N, Roper SD. 2007. The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. *Proc Natl Acad Sci U S A*. 104(15):6436–6441.

Jin YH, Bailey TW, Li BY, Schild JH, Andresen MC. 2004. Purinergic and vanilloid receptor activation releases glutamate from separate cranial afferent terminals in nucleus tractus solitarius. *J Neurosci*. 24(20):4709–4717.

King CT, Garcea M, Spector AC. 2000. Glossopharyngeal nerve regeneration is essential for the complete recovery of quinine-stimulated oromotor rejection behaviors and central patterns of neuronal activity in the nucleus of the solitary tract in the rat. *J Neurosci*. 20(22):8426–8434.

Lasiter PS. 1992. Postnatal development of gustatory recipient zones within the nucleus of the solitary tract. *Brain Res Bull*. 28(5):667–677.

Lundy RF, Norgren R. 2015. Gustatory system. In: Paxinos G, editor. *The rat nervous system*. 4th ed. Academic Press. p. 733–760.

May OL, Erisir A, Hill DL. 2007. Ultrastructure of primary afferent terminals and synapses in the rat nucleus of the solitary tract: comparison among the

greater superficial petrosal, chorda tympani, and glossopharyngeal nerves. *J Comp Neurol*. 502(6):1066–1078.

May OL, Hill DL. 2006. Gustatory terminal field organization and developmental plasticity in the nucleus of the solitary tract revealed through triple-fluorescence labeling. *J Comp Neurol*. 497(4):658–669.

McLean IW, Nakane PK. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J Histochem*. 22:1077–1083.

Mullen RJ, Buck CR, Smith AM. 1992. NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116:201–211.

Takemura M, Sugimoto T, Sakai A. 1987. Topographic organization of central terminal region of different sensory branches of the rat mandibular nerve. *Exp Neurol*. 96(3):540–557.

Takemura M, Sugimoto T, Shigenaga Y. 1991. Difference in central projection of primary afferents innervating facial and intraoral structures in the rat. *Exp Neurol*. 111(3):324–331.

Travers SP. 2002. Quinine and citric acid elicit distinctive Fos-like immunoreactivity in the rat nucleus of the solitary tract. *Am J Physiol Regul Integr Comp Physiol*. 282(6):R1798–R1810.

Travers SP, Norgren R. 1995. Organization of orosensory responses in the nucleus of the solitary tract of rat. *J Neurophysiol*. 73(6):2144–2162.

Travers SP, Travers JB. 2007. Taste-evoked Fos expression in nitrergic neurons in the nucleus of the solitary tract and reticular formation of the rat. *J Comp Neurol*. 500(4):746–760.

Vandenbeuch A, Larson ED, Anderson CB, Smith SA, Ford AP, Finger TE, Kinnamon SC. 2015. Postsynaptic P2X3-containing receptors in gustatory nerve fibres mediate responses to all taste qualities in mice. *J Physiol*. 593(5):1113–1125.

Wang M, Bradley RM. 2010. Properties of GABAergic neurons in the rostral solitary tract nucleus in mice. *J Neurophysiol*. 103(6):3205–3218.

Wang S, Corson J, Hill D, Erisir A. 2012. Postnatal development of chorda tympani axons in the rat nucleus of the solitary tract. *J Comp Neurol*. 520(14):3217–3235.

Watson RE Jr, Wiegand SJ, Clough RW, Hoffman GE. 1986. Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides*. 7(1):155–159.

Whitehead MC. 1986. Anatomy of the gustatory system in the hamster: synaptology of facial afferent terminals in the solitary nucleus. *J Comp Neurol*. 244(1):72–85.

Yao ST, Barden JA, Lawrence AJ. 2001. On the immunohistochemical distribution of ionotropic P2X receptors in the nucleus tractus solitarius of the rat. *Neuroscience*. 108(4):673–685.